

AN INTEGRIN HETERODIMER AND A SUBUNIT THEREOF

FIELD OF THE INVENTION

The present invention relates to a recombinant or isolated integrin heterodimer comprising a subunit $\alpha 10$ and a subunit β , the subunit $\alpha 10$ thereof, homologues and fragments of said integrin and of said subunit $\alpha 10$ having similar biological activity, processes of producing the same, polynucleotides and oligonucleotides encoding the same, vectors and cells comprising the same, binding entities binding specifically to the same, and the use of the same.

BACKGROUND OF THE INVENTION

The integrins are a large family of transmembrane glycoproteins that mediate cell-cell and cell-matrix interactions (1-5). All known members of this superfamily are non-covalently associated heterodimers composed of an α - and a β -subunit. At present, 8 β -subunits ($\beta 1$ - $\beta 8$) (6) and 16 α -subunits ($\alpha 1$ - $\alpha 9$, αv , αM , αL , αX , αIIb , αE and αD) have been characterized (6-21), and these subunits associate to generate more than 20 different integrins. The $\beta 1$ -subunit has been shown to associate with ten different α -subunits, $\alpha 1$ - $\alpha 9$ and αv , and to mediate interactions with extracellular matrix proteins such as collagens, laminins and fibronectin. The major collagen binding integrins are $\alpha 1\beta 1$ and $\alpha 2\beta 1$ (22-25). The integrins $\alpha 3\beta 1$ and $\alpha 9\beta 1$ have also been reported to interact with collagen (26,27) although this interaction is not well understood (28). The extracellular N-terminal regions of the α and β integrin subunits are important in the binding of ligands (29,30). The N-terminal region of the α -subunits is composed of a seven-fold repeated sequence (12,31) containing FG and GAP consensus sequences. The repeats are predicted to fold into a β -propeller domain

(32) with the last three or four repeats containing putative divalent cation binding sites. The α -integrin subunits $\alpha 1$, $\alpha 2$, αD , αE , αL , αM and αX contain a ~200 amino acid inserted domain, the I-domain (A-domain), which
5 shows similarity to sequences in von Willebrand factor, cartilage matrix protein and complement factors C2 and B (33,34). The I-domain is localized between the second and third FG-GAP repeats, it contains a metal ion-dependent
10 adhesion site (MIDAS) and it is involved in binding of ligands (35-38).

Chondrocytes, the only type of cells in cartilage, express a number of different integrins including $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 5\beta 1$, $\alpha 6\beta 1$, $\alpha v\beta 3$, and $\alpha v\beta 5$ (39-41). It has
15 been shown that $\alpha 1\beta 1$ and $\alpha 2\beta 1$ mediate chondrocyte interactions with collagen type II (25) which is one of the major components in cartilage. It has also been shown that $\alpha 2\beta 1$ is a receptor for the cartilage matrix protein chondroadherin (42).

20 SUMMARY OF THE INVENTION

The present invention relates to a novel collagen type II binding integrin, comprising a subunit $\alpha 10$ in association with a subunit β , especially subunit $\beta 1$, but
25 also other β -subunits may be contemplated. In preferred embodiments, this integrin has been isolated from human or bovine articular chondrocytes, and human chondrosarcoma cells.

The invention also encompasses integrin homologues of said integrin, isolated from other species, such as
30 bovine integrin heterodimer comprising a subunit $\alpha 10$ in association with a subunit β , preferably $\beta 1$, as well as homologues isolated from other types of human cells or from cells originating from other species.

The present invention relates in particular to a
35 recombinant or isolated integrin subunit $\alpha 10$ comprising the amino acid sequence shown in SEQ ID No. 1 or SEQ ID No. 2, and homologues and or fragments thereof having the

same biological activity.

The invention further relates to a process of producing a recombinant integrin subunit $\alpha 10$ comprising the amino acid sequence shown in SEQ ID No. 1 or SEQ ID
5 No. 2, or homologues or fragments thereof having similar biological activity, which process comprises the steps of

a) isolating a polynucleotide comprising a nucleotide sequence coding for a integrin subunit $\alpha 10$, or homologues or fragments thereof having similar biological
10 activity,

b) constructing an expression vector comprising the isolated polynucleotide,

c) transforming a host cell with said expression vector,

d) culturing said transformed host cell in a culture medium under conditions suitable for expression of integrin subunit $\alpha 10$, or homologues or fragments thereof having similar biological activity, in said transformed host cell, and, optionally,
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e) isolating the integrin subunit $\alpha 10$, or homologues or fragments thereof having the same biological activity, from said transformed host cell or said culture medium.
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The integrin subunit $\alpha 10$, or homologues or fragments thereof having the same biological activity, can also be provided by isolation from a cell in which they are naturally present.
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The invention also relates to an isolated polynucleotide comprising a nucleotide coding for a integrin subunit $\alpha 10$, or homologues or fragments thereof having similar biological activity, which polynucleotide comprises the nucleotide sequence shown in SEQ ID No. 1 or SEQ ID No. 2, or parts thereof.
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The invention further relates to an isolated polynucleotide or oligonucleotide which hybridises to a DNA or RNA encoding an integrin subunit $\alpha 10$, having the amino acid sequence shown in SEQ ID No. 1 or SEQ ID No. 2, or homologues or fragments thereof, wherein said polyoligo-
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nucleotide or oligonucleotide fails to hybridise to a DNA or RNA encoding the integrin subunit $\alpha 1$.

The invention relates in a further aspect to vectors comprising the above polynucleotides, and to cells containing said vectors and cells that have polynucleotides or oligonucleotides as shown in SEQ ID No. 1 or 2 integrated in their genome.

The invention also relates to binding entities having the capability of binding specifically to the integrin subunit $\alpha 10$ or to homologues or fragments thereof, such as proteins, peptides, carbohydrates, lipids, natural ligands, polyclonal antibodies or monoclonal antibodies.

In a further aspect, the invention relates to a recombinant or isolated integrin heterodimer comprising a subunit $\alpha 10$ and a subunit β , in which the subunit $\alpha 10$ comprises the amino acid sequence shown in SEQ ID No. 1 or SEQ ID No. 2, or homologues or fragments thereof having similar biological activity.

In a preferred embodiment thereof, the subunit β is $\beta 1$.

The invention also relates to a process of producing a recombinant integrin heterodimer comprising a subunit $\alpha 10$ and a subunit β , in which the subunit $\alpha 10$ comprises the amino acid sequence shown in SEQ ID No. 1 or SEQ ID No. 2, which process comprises the steps of

a) isolating one polynucleotide comprising a nucleotide sequence coding for a subunit $\alpha 10$ of an integrin heterodimer and, optionally, another polynucleotide comprising a nucleotide sequence coding for a subunit β of an integrin heterodimer, or for homologues or fragments thereof having similar biological activity,

b) constructing an expression vector comprising said isolated polynucleotide coding for said subunit $\alpha 10$ in combination with an expression vector comprising said isolated nucleotide coding for said subunit β ,

c) transforming a host cell with said expression vectors,

d) culturing said transformed host cell in a culture medium under conditions suitable for expression of an integrin heterodimer comprising a subunit $\alpha 10$ and a subunit β , or homologues or fragments thereof similar biological activity, in said transformed host cell, and, optionally,

e) isolating the integrin heterodimer comprising a subunit $\alpha 10$ and a subunit β , or homologues or fragments thereof having the same biological activity, from said transformed host cell or said culture medium.

The integrin heterodimer, or homologues or fragments thereof having similar biological activity, can also be provided by isolation from a cell in which they are naturally present.

The invention further relates to a cell containing a first vector, said first vector comprising a polynucleotide coding for a subunit $\alpha 10$ of an integrin heterodimer, or for homologues or parts thereof having similar biological activity, which polynucleotide comprises the nucleotide sequence shown in SEQ ID No. 1 or SEQ ID No. 2 or parts thereof, and, optionally, a second vector, said second vector comprising a polynucleotide coding for a subunit β of an integrin heterodimer, or for homologues or fragments thereof.

In still another aspect, the invention relates to binding entities having the capability of binding specifically to the integrin heterodimer comprising a subunit $\alpha 10$ and a subunit β , or to homologues or fragments thereof having similar biological activity, preferably wherein the subunit β is $\beta 1$. Preferred binding entities are proteins, peptides, carbohydrates, lipids, natural ligands, polyclonal antibodies and monoclonal antibodies.

In a further aspect, the invention relates to a fragment of the integrin subunit $\alpha 10$, which fragment is a peptide chosen from the group comprising peptides of

the cytoplasmic domain, the I-domain and the spliced domain.

In one embodiment, said fragment is a peptide comprising the amino acid sequence KLGFFAHKKIPEEEKREEKLEQ.

5 In another embodiment, said fragment comprises the amino acid sequence from about amino acid no. 952 to about amino acid no. 986 of SEQ ID No. 1.

In a further embodiment, said fragment comprises the amino acid sequence from about amino acid No. 140
10 to about amino acid No. 337 in SEQ ID No. 1.

Another embodiment of the invention relates to a polynucleotide or oligonucleotide coding for a fragment of the human integrin subunit $\alpha 10$. In one embodiment this polynucleotide or oligonucleotide codes for a fragment
15 which is a peptide chosen from the group comprising peptides of the cytoplasmic domain, the I-domain and the spliced domain. In further embodiments the polynucleotide or oligonucleotide codes for the fragments defined above.

The invention also relates to binding entities having the capability of binding specifically to a fragment
20 of the integrin subunit $\alpha 10$ as defined above.

The invention also relates to a process of using an integrin subunit $\alpha 10$ comprising the amino acid sequence shown in SEQ ID No. 1 or SEQ ID No. 2, or an integrin
25 heterodimer comprising said subunit $\alpha 10$ and a subunit β , or a homologue or fragment of said integrin or subunit having similar biological activity, as a marker or target molecule of cells or tissues expressing said integrin subunit $\alpha 10$, which cells or tissues are of animal including human origin.
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In an embodiment of this process the fragment is a peptide chosen from the group comprising peptides of the cytoplasmic domain, the I-domain and the spliced domain.

In further embodiments of said process the fragment
35 is a peptide comprising the amino acid sequence KLGFFAHKKIPEEEKREEKLEQ, or a fragment comprising the amino acid sequence from about amino acid No. 952 to

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about amino acid No. 986 of SEQ ID No. 1, or a fragment comprising the amino acid sequence from about amino acid no. 140 to about amino acid no. 337 of SEQ ID no. 1.

The subunit β is preferably $\beta 1$. The cells are preferably chosen from the group comprising chondrocytes, smooth muscle cells, endothelial cells, osteoblasts and fibroblasts.

Said process may be used during pathological conditions involving said subunit $\alpha 10$, such as pathological conditions comprising damage of cartilage, or comprising trauma, rheumatoid arthritis and osteoarthritis.

Said process may be used for detecting the formation of cartilage during embryonal development, or for detecting physiological or therapeutic reparation of cartilage.

Said process may also be used for selection and analysis, or for sorting, isolating or purification of chondrocytes.

A further embodiment of said process is a process for detecting regeneration of cartilage or chondrocytes during transplantation of cartilage or chondrocytes.

A still further embodiment of said process is a process for in vitro studies of differentiation of chondrocytes.

The invention also comprises a process of using binding entities having the capability of binding specifically to an integrin subunit $\alpha 10$ comprising the amino acid sequence shown in SEQ ID No. 1 or SEQ ID No. 2, or an integrin heterodimer comprising said subunit $\alpha 10$ and a subunit β , or to homologues or fragments thereof having similar biological activity, as markers or target molecules of cells or tissues expressing said integrin subunit $\alpha 10$, which cells or tissues are of animal including human origin.

The fragment in said process may be a peptide chosen from the group comprising peptides of the cytoplasmic domain, the I-domain and the spliced domain. In preferred embodiments said fragment is a peptide comprising the

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amino acid sequence KLGFFAHKKIPEEEKREEKLEQ, or a fragment comprising the amino acid sequence from about amino acid No. 952 to about amino acid No. 986 of SEQ ID No. 1, or a fragment comprising the amino acid sequence from about amino acid No. 140 to about amino acid no. 337 of SEQ ID No. 1.

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The process may also be used for detecting the presence of an integrin subunit $\alpha 10$ comprising the amino acid sequence shown in SEQ ID No. 1 or SEQ ID No. 2, or of an integrin heterodimer comprising said subunit $\alpha 10$ and a subunit β , or of homologues or fragments thereof having similar biological activity.

In a further embodiment said process is a process for determining the differentiation-state of cells during embryonic development, angiogenesis, or development of cancer.

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In a still further embodiment this process is a process for detecting the presence of an integrin subunit $\alpha 10$, or of a homologue or fragment of said integrin subunit having similar biological activity, on cells, whereby a polynucleotide or oligonucleotide chosen from the group comprising a polynucleotide or oligonucleotide chosen from the nucleotide sequence shown in SEQ ID No. 1 is used as a marker under hybridisation conditions wherein said polynucleotide or oligonucleotide fails to hybridise to a DNA or RNA encoding an integrin subunit $\alpha 1$. Said cells may be chosen from the group comprising chondrocytes, smooth muscle cells, endothelial cells, osteoblasts and fibroblasts. Said integrin fragment may be a peptide chosen from the group comprising peptides of the cytoplasmic domain, the I-domain and the spliced domain, such as a peptide comprising the amino acid sequence KLGFFAHKKIPEEEKREEKLEQ, or a fragment comprising the amino acid sequence from about amino acid no. 952 to about amino acid no. 986 of SEQ ID No. 1, or a fragment comprising the amino acid sequence from about amino acid No. 140 to about amino acid no. 337 of SEQ ID No. 1.

In a still further embodiment the process is a process for determining the differentiation-state of cells during development, in pathological conditions, in tissue regeneration or in therapeutic and physiological reparation of cartilage. The pathological conditions may be any pathological conditions involving the integrin subunit $\alpha 10$, such as rheumatoid arthritis, osteoarthritis or cancer. The cells may be chosen from the group comprising chondrocytes, smooth muscle cells, endothelial cells, osteoblasts and fibroblasts.

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The invention also relates to a process for determining the differentiation-state of cells during development, in pathological conditions, in tissue regeneration and in therapeutic and physiological reparation of cartilage, whereby a polynucleotide or oligonucleotide chosen from the nucleotide sequence shown in SEQ ID No. 1 is used as a marker under hybridisation conditions wherein said polynucleotide or oligonucleotide fails to hybridise to a DNA or RNA encoding an integrin subunit $\alpha 1$. Embodiments of this aspect comprise a process, whereby said polynucleotide or oligonucleotide is a polynucleotide or oligonucleotide coding for a peptide chosen from the group comprising peptides of the cytoplasmic domain, the I-domain and the spliced domain, such as a polynucleotide or oligonucleotide coding for a peptide comprising the amino acid sequence KLGFFAHKKIPEEEKREEKLEQ, or comprising the amino acid sequence from about amino acid No. 952 to about amino acid no. 986 of SEQ ID No. 1, or the amino acid sequence from about amino acid No. 140 to about amino acid No. 337 of SEQ ID No. 1. Said pathological conditions may be any pathological conditions involving the integrin subunit $\alpha 10$, such as rheumatoid arthritis, osteoarthritis or cancer, or atherosclerosis or inflammation. Said cells may be chosen from the group comprising chondrocytes, smooth muscle cells, endothelial cells, osteoblasts and fibroblasts.

In a further aspect the invention relates to a pharmaceutical composition comprising as an active ingredient a pharmaceutical agent or an antibody which is capable of using an integrin heterodimer comprising a subunit $\alpha 10$ and a subunit β , or the subunit $\alpha 10$ thereof, or a homologue or fragment of said integrin or subunit $\alpha 10$ having similar biological activity, as a target molecule. An embodiment of said pharmaceutical composition is intended for use in stimulating, inhibiting or blocking the formation of cartilage, bone or blood vessels. A further embodiment comprises a pharmaceutical composition for use in preventing adhesion between tendon/ligaments and the surrounding tissue after infection, inflammation and after surgical intervention where adhesion impairs the function of the tissue.

The invention is also related to a vaccine comprising as an active ingredient an integrin heterodimer comprising a subunit $\alpha 10$ and a subunit β , or the subunit $\alpha 10$ thereof, or a homologue or fragment of said integrin or subunit $\alpha 10$, or DNA or RNA coding for said integrin subunit $\alpha 10$.

A further aspect of the invention is related to the use of the integrin subunit $\alpha 10$ as defined above as a marker or target in transplantation of cartilage or chondrocytes.

A still further aspect of the invention is related to a method of using binding entities having the capability of binding specifically to an integrin subunit $\alpha 10$ comprising the amino acid sequence shown in SEQ ID No. 1 or SEQ ID No. 2, or an integrin heterodimer comprising said subunit $\alpha 10$ and a subunit β , or to homologues or fragments thereof having similar biological activity, for promoting adhesion of chondrocytes and/or osteoblasts to surfaces of implants to stimulate osseointegration.

The invention is also related to the use of an integrin subunit $\alpha 10$ or an integrin heterodimer comprising said subunit $\alpha 10$ and a subunit β as a target for anti-

adhesive drugs or molecules in tendon, ligament, skeletal muscle or other tissues where adhesion impairs the function of the tissue.

The invention also relates to a method of stimulating, inhibiting or blocking the formation of cartilage or bone, comprising administration to a subject a suitable amount of a pharmaceutical agent or an antibody which is capable of using an integrin heterodimer comprising a subunit $\alpha 10$ and a subunit β , or the subunit $\alpha 10$ thereof, or a homologue or fragment of said integrin or subunit $\alpha 10$ having similar biological activity, as a target molecule.

In another embodiment the invention is related to a method of preventing adhesion between tendon/ligaments and the surrounding tissue after infection, inflammation and after surgical intervention where adhesion impairs the function of the tissue, comprising administration to a subject a suitable amount of a pharmaceutical agent or an antibody which is capable of using a integrin heterodimer comprising a subunit $\alpha 10$ and a subunit β , or the subunit $\alpha 10$ thereof, or a homologue or fragment of said integrin or subunit $\alpha 10$ having similar biological activity, as a target molecule.

The invention also relates to a method of stimulating extracellular matrix synthesis and repair by activation or blockage of an integrin heterodimer comprising a subunit $\alpha 10$ and a subunit β , or of the subunit $\alpha 10$ thereof, or of a homologue or fragment of said integrin or subunit $\alpha 10$ having similar biological activity.

In a further aspect the invention relates to a method of in vitro detecting the presence of integrin binding entities, comprising interaction of an integrin heterodimer comprising a subunit $\alpha 10$ and a subunit β , or the subunit $\alpha 10$ thereof, or a homologue or fragment of said integrin or subunit, with a sample, thereby causing said integrin, subunit $\alpha 10$, or homologue or fragment thereof having similar biological activity, to modulate

the binding to its natural ligand or other integrin binding proteins present in said sample.

The invention also relates to a method of in vitro studying consequences of the interaction of a human
5 heterodimer integrin comprising a subunit $\alpha 10$ and a subunit β , or the subunit $\alpha 10$ thereof, or a homologue or fragment of said integrin or subunit, with an integrin binding entity and thereby initiate a cellular reaction. Said consequences may be measured as alterations in cellular functions.
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A still further aspect of the inventions relates to a method of using DNA or RNA encoding an integrin subunit $\alpha 10$ or homologues or fragments thereof as a molecular target. In an embodiment of this aspect, a polynucleotide
15 or oligonucleotide hybridises to the DNA or RNA encoding an integrin subunit $\alpha 10$ or homologues or fragments thereof, whereby said polynucleotide or oligonucleotide fails to hybridise to a DNA or RNA encoding an integrin subunit $\alpha 1$.

20 The invention also relates to a method of using a human heterodimer integrin comprising a subunit $\alpha 10$ and a subunit β , or the subunit $\alpha 10$ thereof, or a homologue or fragment of said integrin or subunit, or a DNA or RNA encoding an integrin subunit $\alpha 10$ or homologues or fragments thereof, as a marker or target molecule during
25 angiogenesis.

BRIEF DESCRIPTION OF THE FIGURES

Fig.1 Affinity purification of the $\alpha 10$ integrin subunit on collagen type II-Sepharose.

30 Fig. 2. Amino acid sequences of peptides from the bovine $\alpha 10$ integrin subunit.

Fig. 3a. Affinitypurification and immunoprecipitation of the integrin subunit $\alpha 10$ from bovine chondrocytes.

35 Fig. 3b. Affinitypurification and immunoprecipitation of the integrin subunit $\alpha 10$ from human chondrocytes.

Fig. 3c. Affinitypurification and immunoprecipitation of the integrin subunit $\alpha 10$ from human chondrosarcoma cells.

Fig. 4. A 900 bp PCR-fragment corresponding to the
5 bovine integrin subunit $\alpha 10$

Fig. 5. Schematic map of the three overlapping $\alpha 10$ clones.

Fig. 6. Nucleotide sequence and deduced amino acid sequence of the human $\alpha 10$ integrin subunit.

10 Fig. 7. Northern blot of integrin $\alpha 10$ mRNA.

Fig. 8 Immunoprecipitation of the $\alpha 10$ integrin subunit from human chondrocytes using antibodies against the cytoplasmic domain of $\alpha 10$ (a). Western blot of the $\alpha 10$ associated β -chain (b).

15 Fig. 9. Immunostaining of $\alpha 10$ integrin in human articular cartilage.

Fig. 10 Immunostaining of $\alpha 10$ integrin in 3 day mouse limb cartilage.

20 Fig 11. Immunostaining of $\alpha 10$ integrin in 13.5 day mouse embryo.

Fig 12. Hybridisation of $\alpha 10$ mRNA in various human tissues.

25 Fig. 13 Immunostaining of fascia around tendon (a), skeletal muscle (b) and heart valves (c) in 3 day mouse limb.

Fig. 14. PCR fragments corresponding to $\alpha 10$ integrin subunit from human chondrocytes, human endothelial cells, human fibroblasts and rat tendon.

30 Fig 15. Partial genomic nucleotide sequence of the human integrin subunit $\alpha 10$.

Fig 16. Upregulation of $\alpha 10$ integrin subunit in chondrocytes cultured in alginate.

35 Fig 17. Immunoprecipitation of the $\alpha 10$ integrin subunit from human smooth muscle cells

DETAILED DESCRIPTION OF THE INVENTION

The present invention demonstrate that human and

bovine chondrocytes express a novel, collagen type II-binding integrin in the $\beta 1$ -family. An earlier study presented some evidence for that human chondrosarcoma cells also express this integrin (25). Immunoprecipitation experiments using antibodies against the integrin subunit $\beta 1$ revealed that this novel α -integrin subunit had an apparent molecular weight (M_r) of approximately 160 kDa under reducing conditions, and was slightly larger than the $\alpha 2$ integrin subunit. To isolate this α -subunit collagen type II-binding proteins were affinity purified from bovine chondrocytes. The chondrocyte lysate was first applied to a fibronectin-Sepharose precolumn and the flow through was then applied to a collagen type II-Sepharose column. A protein with M_r of approximately 160 kD was specifically eluted with EDTA from the collagen column but not from the fibronectin column. The M_r of this protein corresponded with the M_r of the unidentified $\beta 1$ -related integrin subunit. The 160 kD protein band was excised from the SDS-PAGE gel, digested with trypsin and the amino acid sequences of the isolated peptides were analysed.

Primers corresponding to isolated peptides amplified a 900 bp PCR-fragment from bovine cDNA which was cloned, sequenced and used for screening of a human articular chondrocyte λ ZapII cDNA library to obtain the human integrin α -subunit homologue. Two overlapping clones, hc1 and hc2 were isolated, subcloned and sequenced. These clones contained 2/3 of the nucleotide sequence including the 3' end of the cDNA. A third clone which contained the 5' end of the $\alpha 10$ cDNA, was obtained using the RACE technique. Sequence analysis of the 160 kD protein sequence showed that it was a member of the integrin α -subunit family and the protein was named $\alpha 10$.

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The deduced amino acid sequence of $\alpha 10$ was found to share the general structure of the integrin α -subunits described in previously published reports (6-21). The large extracellular N-terminal part of $\alpha 10$ contains a

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seven-fold repeated sequence which was recently predicted to fold into a β -propeller domain (32). The integrin subunit $\alpha 10$ contains three putative divalent cation-binding sites (DxD/NxD/NxxxD) (53), a single spanning transmembrane domain and a short cytoplasmic domain. In contrast to most α -integrin subunits the cytoplasmic domain of $\alpha 10$ does not contain the conserved sequence KxGFF (R/K) R. The predicted amino acid sequence in $\alpha 10$ is KLGFFAH. Several reports indicate that the integrin cytoplasmic domains are crucial in signal transduction (54) and that membrane-proximal regions of both α - and β -integrin cytoplasmic domains are involved in modulating conformation and affinity state of integrins (55-57). It is suggested that the GFFKR motif in α -chains are important for association of integrin subunits and for transport of the integrin to the plasma membrane (58). The KxGFFKR domain has been shown to interact with the intracellular protein calreticulin (59) and interestingly, calreticulin-null embryonic stem cells are deficient in integrin-mediated cell adhesion (60). It is therefor possible that the sequence KLGFFAH in $\alpha 10$ have a key function in regulating the affinity between $\alpha 10\beta 1$ and matrix proteins.

Integrin α subunits are known to share an overall identity of 20-40% (61). Sequence analysis showed that the $\alpha 10$ subunit is most closely related to the I-domain containing α -subunits with the highest identity to $\alpha 1$ (37%) and $\alpha 2$ (35%). The integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ are known receptors for both collagens and laminins (24;62;63) and we have also recently demonstrated that $\alpha 2\beta 1$ interacts with the cartilage matrix protein chondroadherin (42). Since $\alpha 10\beta 1$ was isolated on a collagen type II-Sepharose, we know that collagen type II is a ligand for $\alpha 10\beta 1$. We have also shown by affinity purification experiments that $\alpha 10\beta 1$ interacts with collagen type I but it remains to be seen whether laminin or chondroadherin are also ligands for this integrin.

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- The $\alpha 10$ associated β -chain migrated as the $\beta 1$ integrin subunit both under reducing and non-reducing conditions. To verify that the $\alpha 10$ associated β -chain indeed is $\beta 1$, chondrocyte lysates were immunoprecipitated with antibodies against $\alpha 10$ or $\beta 1$ followed by Western blot using antibodies against the $\beta 1$ -subunit. These results clearly demonstrated that $\alpha 10$ is a member of the $\beta 1$ -integrin family. However, the possibility that $\alpha 10$ combine also with other β -chains can not be excluded..
- A polyclonal peptide antibody raised against the cytoplasmic domain of $\alpha 10$ precipitated two protein bands with M_r of approximately 160 kD ($\alpha 10$) and 125 kD ($\beta 1$) under reducing conditions. Immunohistochemistry using the $\alpha 10$ -antibody showed staining of the chondrocytes in tissue sections of human articular cartilage. The antibody staining was clearly specific since preincubation of the antibody with the $\alpha 10$ -peptide completely abolished the staining. Immunohistochemical staining of mouse limb sections from embryonic tissue demonstrated that $\alpha 10$ is upregulated during condensation of the mesenchyme. This indicate that the integrin subunit $\alpha 10$ is important during the formation of cartilage. In 3 day old mice $\alpha 10$ was found to be the dominating collagen binding integrin subunit which point to that $\alpha 10$ has a key function in maintaining normal cartilage functions.
- Expression studies on the protein and mRNA level show that the distribution of $\alpha 10$ is rather restrictive. Immunohistochemistry analyses have shown that $\alpha 10$ integrin subunit is mainly expressed in cartilage but it is also found in perichondrium, periosteum, ossification groove of Ranvier, in fascia surrounding tendon and skeletal muscle and in the tendon-like structures in the heart valves. This distribution point to that $\alpha 10$ integrin subunit is present also on fibroblasts and osteoblasts. PCR amplification of cDNA from different cell types revealed the presence of an alternatively spliced $\alpha 10$ integrin subunit. This spliced $\alpha 10$ was domi-

nating in fibroblasts which suggests that $\alpha 10$ in fibroblasts may have a different function compared to $\alpha 10$ present on chondrocytes.

Expression of the integrin subunit $\alpha 10$ was found to
5 decrease when chondrocytes were cultured in monolayer. In contrast, the expression of $\alpha 10$ was found to increase when the cells were cultured in alginate beads. Since the latter culturing model is known to preserve the phenotype of chondrocytes the results suggest that $\alpha 10$ can function
10 as marker for a differentiated chondrocyte.

Adhesion between tendon/ligaments and the surrounding tissue is a well-known problem after infection, injury and after surgical intervention. Adhesion between tendon and tendon sheets impairs the gliding function and
15 cause considerable problems especially during healing of tendons in e.g. the hand and fingers leading to functional incapacity. The localisation of the $\alpha 10$ integrin subunit in the fascia of tendon and skeletal muscle makes $\alpha 10$ a possible target for drugs and molecules with anti-
20 adhesive properties that could prevent impairment of the function of tendon/ligament. The integrin subunit $\alpha 10$ can also be a target for drugs or molecules with anti-adhesive properties in other tissues where adhesion is a problem.

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EXAMPLES

Example 1

Affinity purification of the $\alpha 10$ integrin subunit on
30 collagen type II-Sepharose.

Materials and Methods

Bovine chondrocytes, human chondrocytes or human chondrosarcoma cells were isolated as described earlier [Holmvalle et al, Exp Cell Res, 221, 496-503 (1995),
35 Camper et al, JBC, 273, 20383-20389 (1998)]. A Triton X-100 lysate of bovine chondrocytes was applied to a fibronectin-Sepharose precolumn followed by a collagen

type II-Sepharose column and the integrin subunit $\alpha 10$ was eluted from the collagen type II-column by EDTA (Camper et al, JBC, 273, 20383-20389 (1998). The eluted proteins were precipitated by methanol/chloroform, separated by
5 SDS-PAGE under reducing conditions and stained with Coomassie blue. (Camper et al, JBC, 273, 20383-20389 (1998). Peptides from the $\alpha 10$ protein band were isolated by in-gel digestion with a trypsin and phase liquid chromatography and sequenced by Edman degradation (Camper et
10 al, JBC, 273, 20383-20389 (1998).

Results

Fig 1 shows EDTA-eluted proteins from the fibronectin-Sepharose (A), flow-through from the collagen type II-Sepharose column (B) and EDTA-eluted proteins from the
15 collagen type II-Sepharose (C). The $\alpha 10$ integrin subunit (160 kDa) which was specifically eluted from the collagen type II column is indicated with an arrow. Figure 2 shows the amino acid sequences of six peptides that were isolated from the bovine integrin subunit $\alpha 10$. Figures 3 a,
20 b, and c show that the $\alpha 10$ integrin subunit is present on bovine chondrocytes (3a), human chondrocytes (3b) and human chondrosarcoma cells (3c). The affinity for collagen type II, the coprecipitation with $\beta 1$ -integrin subunit and the molecular weight of 160 kDa under reducing conditions identify the $\alpha 10$ integrin subunit on the different
25 cells. These results show that $\alpha 10$ can be isolated from chondrocytes and from chondrosarcoma cells.

Example 2

30 Amplification of PCR fragment corresponding to bovine $\alpha 10$ integrin subunit.

Materials and methods

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The degenerate primers GAY AAY ACI GCI CAR AC (DNTAQT, forward) and TIA TIS WRT GRT GIG GYT (EPHHSI,
35 reverse) were used in PCR (Camper et al, JBC, 273, 20383-20389 (1998) to amplify the nucleotide sequence corresponding to the bovine peptide 1 (Figure 2). A 900 bp

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PCR-fragment was then amplified from bovine cDNA using an internal specific primer TCA GCC TAC ATT CAG TAT (SAYIQY, forward) corresponding to the cloned nucleotide sequence of peptide 1 together with the degenerate primer ICK RTC CCA RTG ICC IGG (PGHWDR, reverse) corresponding to the bovine peptide 2 (Figure 2). Mixed bases were used in positions that were twofold degenerate and inosines were used in positions that are three- or fourfold degenerate. mRNA isolation and cDNA synthesis was done as earlier described (Camper et al, JBC, 273, 20383-20389 (1998)). The purified fragment was cloned, purified and sequenced as earlier described (Camper et al, JBC, 273, 20383-20389 (1998)).

Results

15 The nucleotide sequence of peptide 1 (Figure 2) was obtained by PCR-amplification, cloning and sequencing of bovine cDNA. From this nucleotide sequence an exact primer was designed and applied in PCR-amplification with degenerate primers corresponding to peptides 20 2-6 (Figure 2). Primers corresponding to peptides 1 and 2 amplified a 900 bp PCR-fragment from bovine cDNA (Figure 4).

Example 3

25 Cloning and sequence analysis of the human $\alpha 10$ integrin subunit

Material and methods

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The cloned 900bp PCR-fragment, corresponding to bovine $\alpha 10$ -integrin, was digoxigenin-labelled according to the DIG DNA labelling kit (Boehringer Mannheim) and used as a probe for screening of a human articular chondrocyte λ ZapII cDNA library (provided by Michael Bayliss, The Royal Veterinary Basic Sciences, London, UK) (52). Positive clones containing the pBluescript SK+ plasmid with the cDNA insert were rescued from the ZAP vector by *in vivo* excision as described in the ZAP-cDNA[®] synthesis kit (Stratagene). Selected plasmids were purified and

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sequenced as described earlier (Camper et al, JBC, 273, 20383-20389 (1998)) using T3, T7 and internal specific primers. To obtain cDNA that encoded the 5' end of $\alpha 10$ we designed the primer AAC TCG TCT TCC AGT GCC ATT CGT GGG (reverse; residue 1254-1280 in $\alpha 10$ cDNA) and used it for rapid amplification of the cDNA 5' end (RACE) as described in the Marathon™ cDNA Amplification kit (Clontech INC., Palo Alto, CA).

Results

Two overlapping clones, hc1 and hc2 (Figure 5), were isolated, subcloned and sequenced. These clones contained 2/3 of the nucleotide sequence including the 3' end of the cDNA. A third clone (racel; Figure 5), which contained the 5' end of the $\alpha 10$ cDNA, was obtained using the RACE technique. From these three overlapping clones of $\alpha 10$ cDNA, 3884 nucleotides were sequenced. The nucleotide sequence and deduced amino acid sequence is shown in Figure 6. The sequence contains a 3504-nucleotide open reading frame that is predicted to encode a 1167 amino acid mature protein. The signal peptide cleavage site is marked with an arrow, human homologues to bovine peptide sequences are underlined and the I-domain is boxed. Metal ion binding sites are indicated with a broken underline, potential N-glycosylation sites are indicated by an asterisk and the putative transmembrane domain is double underlined. The normally conserved cytoplasmic sequence is indicated by a dot and dashed broken underline.

Sequence analysis demonstrate that $\alpha 10$ is a member of the integrin α -subunit family.

Example 4

Identification of a clone containing a splice variant of $\alpha 10$

One clone which was isolated from the human chondrocyte library (see Example 3) contained a sequence that was identical to the sequence of $\alpha 10$ integrin subunit except that the nucleotides between nt positions

2942 and 3055 were deleted. The splice variant of $\alpha 10$ was verified in PCR experiment using primers flanking the splice region (see figure 14).

5 Example 5

Identification of $\alpha 10$ integrin subunit by Northern blot

Material and methods

Bovine chondrocyte mRNA was purified using a
10 QuickPrep® Micro mRNA Purification Kit (Pharmacia Biotech, Uppsala, Sweden), separated on a 1% agarose-formaldehyde gel, transferred to nylon membranes and immobilised by UV crosslinking. cDNA-probes were ^{32}P -labelled with Random Primed DNA Labeling Kit (Boehringer Mannheim). Filters
15 were prehybridised for 2-4 hours at 42°C in 5x SSE, 5x Denharts solution, 0.1 % SDS, 50 $\mu\text{g}/\text{ml}$ salmon sperm DNA and 50% formamide and then hybridised over night at 42°C with the same solution containing the specific probe ($0.5\text{--}1 \times 10^6$ cpm/ml). Specifically bound cDNA-
20 probes were analysed using the phosphoimager system (Fuji). Filters were stripped by washing in 0.1% SDS, for 1 hour at 80°C prior to re-probing. The $\alpha 10$ -integrin cDNA-probe was isolated from the rac1-containing plasmid using the restriction enzymes BamHI (GIBCO BRL) and NcoI
25 (Boehringer Mannheim). The rat $\beta 1$ -integrin cDNA probe was a kind gift from Staffan Johansson, Uppsala, Sweden.

Results

Northern blot analysis of mRNA from bovine chondrocytes showed that a human $\alpha 10$ cDNA-probe hybridised with
30 a single mRNA of approximately 5.4 kb (Figure 7). As a comparison, a cDNA-probe corresponding to the integrin subunit $\alpha 1$ was used. This cDNA-probe hybridised a mRNA-band of approximately 3.5 kb on the same filter. These results show that a cDNA-probe against $\alpha 10$ can be used to
35 identify the $\alpha 10$ integrin subunit on the mRNA level.

Example 6

Preparation of antibodies against the integrin subunit $\alpha 10$

A peptide corresponding to part of the $\alpha 10$ cytoplasmic domain, Ckkipееееkreekle (see figure 6) was synthesised and conjugated to keyhole limpet hemocyanin (KLH). Rabbits were immunised with the peptide-KLH conjugate to generate antiserum against the integrin subunit $\alpha 10$. Antibodies recognising $\alpha 10$ were affinity purified on an peptide-coupled column (Innovagen AB).

Example 7

Immunoprecipitation of the integrin subunit $\alpha 10$ from chondrocytes

15 Material and methods

Human chondrocytes were ^{125}I -labelled, lysed with Triton X-100 and immunoprecipitated as earlier described (Holmval1 et al, Exp Cell Res, 221, 496-503 (1995), Camper et al, JBC, 273, 20383-20389 (1998)). Triton X-100 lysates of ^{125}I -labeled human chondrocytes were immunoprecipitated with polyclonal antibodies against the integrin subunits $\beta 1$, $\alpha 1$, $\alpha 2$, $\alpha 3$ or $\alpha 10$. The immunoprecipitated proteins were separated by SDS-PAGE (4-12%) under non-reducing conditions and visualised using a phosphor imager. Triton X-100 lysates of human chondrocytes immunoprecipitated with $\alpha 10$ or $\beta 1$ were separated by SDS-PAGE (8%) under non-reducing conditions and analysed by Western blot using the polyclonal $\beta 1$ antibody and chemiluminescent detection as described in Camper et al, JBC, 273, 20383-20389 (1998).

30 Results

The polyclonal peptide antibody, raised against the cytoplasmic domain of $\alpha 10$, precipitated two protein bands with Mr of approximately 160 kD ($\alpha 10$) and 125 kD ($\beta 1$) under reducing conditions. The $\alpha 10$ associated β -chain migrated as the $\beta 1$ integrin subunit (Figure 8a). To verify that the $\alpha 10$ associated β -chain in chondrocytes

indeed is $\beta 1$, chondrocyte lysates were immunoprecipitated with antibodies against $\alpha 10$ or $\beta 1$ followed by Western blot using antibodies against the $\beta 1$ -subunit (Figure 8b). These results clearly demonstrated that $\alpha 10$ is a member of the $\beta 1$ -integrin family. However, the results do not exclude the possibility that $\alpha 10$ can associate with other β -chains in other situations.

Example 8

10 Immunohistochemical staining of the integrin subunit $\alpha 10$ in human and mouse cartilage

Material and methods

Frozen sections of adult cartilage (trochlear groove) obtained during surgery (provided by Anders Lindahl, Salgrenska Hospital, Gothenburg, Sweden and frozen sections from of 3 day old mouse limb were fixed and prepared for immunohistochemistry as earlier described (Camper et al, JBC, 273, 20383-20389 (1998)). Expression of $\alpha 10$ integrin subunit was analysed using the polyclonal antibody against the cytoplasmic domain as a primary antibody (see Example 6) and a secondary antibody conjugated to peroxidase.

Results

Figures 9 show immunostaining of human adult articular cartilage.

The $\alpha 10$ -antibody recognising the cytoplasmic domain of $\alpha 10$ stained the chondrocytes in tissue sections of human articular cartilage (A). The staining was depleted when the antibody was preincubated with the $\alpha 10$ -peptide (B). A control antibody recognising the $\alpha 9$ integrin subunit did not bind to the chondrocyte (C).

Figures 10 shows that the $\alpha 10$ antibody stain the majority of chondrocytes in the growing bone anlage (a and b). The $\alpha 10$ antibody also recognised cells in the ossification groove of Ranvier (b), especially the osteoblast in the bone bark which are lining the cartilage in the metaphys are highly positive for $\alpha 10$. The

cells in the ossification groove of Ranvier are believed to be important for the growth in diameter of the bone. The integrin subunit $\alpha 10$ is also highly expressed in perichondrium and periosteum. Cell in these tissues are likely important in the repair of the cartilage tissue. The described localisation of the integrin subunit $\alpha 10$ suggest that this integrin is important for the function of the cartilage tissue.

10 Example 9

Immunohistochemical staining of the integrin subunit $\alpha 10$ during mouse development

Material and methods

Frozen sections from mouse embryos (13.5 days) were investigated for expression of $\alpha 10$ by immunohistochemistry as described in Camper et al, JBC, 273, 20383-20389 (1998). Expression of $\alpha 10$ integrin subunit was analysed using the polyclonal antibody against the cytoplasmic domain as a primary antibody (see Example 6) and a secondary antibody conjugated to peroxidase. The embryo sections were also investigated for expression of integrin subunit $\alpha 1$ (monoclonal antibody from Pharmingen) and collagen type II (monoclonal antibody, kind gift from Dr John Mo, Lund University, Sweden).

25 Results

Figure 11 show that $\alpha 10$ integrin subunit is unregulated in the limb when the mesenchymal cells undergo condensation to form cartilage (a). Especially the edge of the newly formed cartilage has high expression of $\alpha 10$. The formation of cartilage is verified by the high expression of the cartilage specific collagen type II (b). The control antibody against $\alpha 1$ integrin subunit showed only weak expression on the cartilage (c). In other experiments expression of $\alpha 10$ was found in all cartilage containing tissues in the 3 day old mouse including limbs, ribs and vertebrae. The upregulation of $\alpha 10$ during formation of cartilage suggest that this integrin subunit is

important both in the development of cartilage and bone and in the repair of damaged cartilage tissue.

Example 10

- 5 mRNA expression of $\alpha 10$ in tissues other than articular cartilage

Material and methods

- Expression of $\alpha 10$ integrin subunit was examined on the mRNA level in different human tissues. A Northern dot blot with immobilised mRNA from the listed tissues in Figure 12 was hybridised with an $\alpha 10$ integrin cDNA probe isolated from the race 1-containing plasmid using the restriction enzymes *Bam*H1 and *Nco*1. The degree of hybridisation was analysed using a phospho imager. The following symbols denote mRNA level in increasing order: -, +, ++, +++, +++++.

Results

- Analysis of the hybridised mRNA showed that $\alpha 10$ was expressed in aorta, trachea, spinal cord, heart, lung, and kidney (Figure 12). All other tissues appeared negative for $\alpha 10$ expression. These results point to a restricted distribution of the $\alpha 10$ integrin subunit.

Example 11

- 25 Immunohistochemical staining of $\alpha 10$ in fascia around tendon and skeletal muscle and in tendon structures in heart valves.

Materials and methods

- Frozen sections of adult cartilage (trochlear groove) obtained during surgery (provided by Anders Lindahl, Salgrenska Hospital, Gothenburg, Sweden and frozen sections from of 3 day old mouse limb were fixed and prepared for immunohistochemistry as earlier described (Camper et al, JBC, 273, 20383-20389 (1998)). Expression of $\alpha 10$ integrin subunit was analysed using the polyclonal antibody against the cytoplasmic domain as a pri-

mary antibody (see Example 6) and a secondary antibody conjugated to peroxidase.

Results

As shown in figures 13 expression of $\alpha 10$ was found
5 in the fascia surrounding tendon (a) and skeletal muscle
(b) and in the tendon structures in the heart valves (c).
This localisation suggest that $\alpha 10$ can bind to other
matrix molecules in addition to the cartilage specific
collagen type II. The localisation of the integrin $\alpha 10$ on
10 the surface of tendons indicate that $\alpha 10$ can be involved
in unwanted adhesion that often occurs between tendon/
ligaments and the surrounding tissue after infection,
injury or after surgery.

15 Example 12

mRNA expression of $\alpha 10$ integrin subunit in chondro-
cytes, endothelial cells and fibroblasts.

Material and methods

Isolation of mRNA, synthesis of cDNA and PCR ampli-
20 fication was done as earlier described (Camper et al,
JBC, 273, 20383-20389 (1998)).

Results

Figure 14 shows PCR amplification of $\alpha 10$ cDNA from
human articular chondrocytes (lanes A6 and B1), human
25 umbilical vein endothelial cells (lane A2), human fibro-
blasts (lane A4) and rat tendon (Fig 14b, lane B2). Lanes
1, 3, and 5 in figure 14 A show amplified fragments cor-
responding to the integrin subunit $\alpha 2$ in endothelial
cells, fibroblasts and chondrocytes, respectively. cDNA-
30 primers corresponding to the $\alpha 10$ sequence positions nt
2919-2943 (forward) and nt 3554-3578 (reverse) (see
Figure 6) were used to amplify $\alpha 10$ cDNA from the diffe-
rent cells. The figure shows that $\alpha 10$ was amplified in
all three cell types. Two fragments of $\alpha 10$ was amplified
35 which represent the intact form of $\alpha 10$ (larger fragment)
and a splice variant (smaller fragment). The larger frag-

ment was dominating in chondrocytes while the smaller fragment was more pronounced in tendon (B2).

Example 13

5 Construction of $\alpha 10$ mammalian expression vector.

The full length protein coding sequence of $\alpha 10$ (combined from 3 clones, see figure 6) was inserted into the mammalian expression vector, pcDNA3.1/Zeo (Invitrogen). The vector contains SV40 promoter and Zeosin selection
10 sequence. The $\alpha 10$ containing expression vector was transfected into cells that express the $\beta 1$ -integrin subunit but lack expression of the $\alpha 10$ subunit. Expression of the $\alpha 10$ integrin subunit on the cell surface can be analysed by immunoprecipitation and/or flow cytometry using anti-
15 bodies specific for $\alpha 10$. The ligand binding capacity and the function of the inserted $\alpha 10$ integrin subunit can be demonstrated in cell adhesion experiment and in signalling experiments.

20 Example 14

Construction of mammalian expression vector containing a splice variant of $\alpha 10$.

The full length protein coding sequence of the splice variant of $\alpha 10$ (nt 2942-nt3055 deleted) was
25 inserted into the mammalian expression vector pcDNA3 (see Example 13). Expression and function of the splice variant can be analysed as described in example 13 and compared with the intact $\alpha 10$ integrin subunit.

30 Example 15

Partial isolation and characterisation of the $\alpha 10$ integrin genomic DNA

Material and methods

Human $\alpha 10$ cDNA, isolated from the racel-containing
35 plasmid using the restriction enzymes *Bam*HI (GIBCO BRL) and *Nco*I (Boehringer Mannheim), was 32 P-labelled and used as a probe for screening of a mouse 129 cosmid library

(provided by Reinhard Fässler, Lund University). Positive clones were isolated and subcloned. Selected plasmids were purified and sequenced as described earlier (Camper et al, JBC, 273, 20383-20389 (1998)) using T3, T7 and internal specific primers. Primers corresponding to mouse genomic DNA were then constructed and used in PCR to amplify and identify the genomic sequence of $\alpha 10$ from the cosmid clones.

Results

Figure 15 shows 7958 nt of the $\alpha 10$ gene. This partial genomic DNA sequence of $\alpha 10$ integrin contains 8 exons, and a Kozak sequence. The mouse genomic $\alpha 10$ sequence was used to generate a targeting vector for knockout experiments.

Example 16

Upregulation of $\alpha 10$ integrin subunit in chondrocytes cultured in alginate beads

Material and methods

Human chondrocytes cultured in monolayer for 2 weeks were detached with trypsin-EDTA and introduced into alginate beads. Chondrocytes cultured in alginate are known to preserve their phenotype while chondrocytes cultured in monolayer are dedifferentiated. After 11 days chondrocytes cultured either in alginate or on monolayer were isolated and surface labelled with ^{125}I . The $\alpha 10$ integrin subunit was then immunoprecipitated with polyclonal antibodies recognising the cytoplasmic domain of $\alpha 10$ (see Example 6 and Camper et al, JBC, 273, 20383-20389 (1998)).

Results

As shown in figure 16 chondrocytes cultured in alginate beads (lanes 3 and 4) upregulated their protein expression of $\alpha 10\beta 1$. This was in contrast to chondrocytes cultured in monolayer (lanes 1 and 2) which had a very low expression of $\alpha 10\beta 1$. Immunoprecipitation with ab control antibody is shown in lanes 1 and 3. It is known that

chondrocytes preserve their cartilage specific matrix production in alginate cultures but not in monolayer culture which point to that alginate preserve the phenotype of chondrocytes. These results support that $\alpha 10$ integrin subunit can be used as a marker for differentiated chondrocytes.

Example 17

Immunoprecipitation of the $\alpha 10$ integrin subunit from human smooth muscle cells.

Material and methods

Human smooth muscle cells were isolated from human aorta. After one week in culture the cells were ^{125}I -labelled, lysed and immunoprecipitated with antibodies against the integrin subunit $\beta 1$ (lane 1), $\alpha 1$ (lane 2), $\alpha 2$ (lane 3), $\alpha 10$ (lane 4), $\alpha 3$ (lane 5), control (lane 6) (Figure 17). The experiment was done as described in Example 7.

Results

The $\alpha 10$ antibody precipitated two bands from the smooth muscle cells corresponding to the $\alpha 10$ and the $\beta 1$ integrin subunit (Fig. 17).

Example 18

Construction of bacterial expression vector containing sequence for $\alpha 10$ splice region.

sub B7
A plasmid for intracellular expression in E. coli of the alternatively spliced region (amino acid pos. 952-986, SEQ. ID 1) was constructed as described. The alternatively spliced region were back-translated using the E. coli high frequency codon table, creating a cDNA sequence of 96% identity with the original sequence (SEQ. ID 1 nucleotide pos 2940-3044). Using sequence overlap extension (Horton et al., Biotechniques 8:528, 1990) primer $\alpha 10\text{pfor}$ (tab. I) and $\alpha 10\text{prev}$ (tab. I) was used to generate a double stranded fragment encoding the $\alpha 10$ amino acid sequence. This fragment was used as a PCR

template with primers $\alpha 10pfor2$ (tab. I) and $\alpha 10prev2$ (tab. I) in order to generate restriction enzyme site for sub-cloning in a pET vector containing the Z-domain of staphylococcal protein A, creating a fusion of the $\alpha 10$ spliced region with the amino terminal of the Z-domain with trombin cleavage site residing in-between. The fragment generated in the second PCR reaction is shown (SEQ ID No. 3) also indicating the unique restriction enzymes used for sub-cloning in the expression vector.

Table I

$\alpha 10pfor$	5' - G TTCAGAACCTGGGTTGCTACGTTGTTTCCGGTCTGATCATCTCCGC TCTGCTGCCGGCTGT-3'
$\alpha 10pfor2$	5' -GGGGCATATGGTTCAGAACCTGGGTTGCTACGTTG-3'
$\alpha 10prev$	5' - GATAACCTGGGACAAGCTTAGGAAGTAGTTACCACCGTGAGCAACAG CCGGCAGCAGAGCGGA-3'
$\alpha 10prev2$	5' - GGGGGGATCCGCGCGGCACCAGGCCGCTGATAACCTGGGACAAGCTT AGGAAGT-3'

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